Data Evaluation Report on the Long-Term Impact of Clothianidin-Treated Canola Seed on Honey Bees (Hive Study)

PMRA Submission Number {.....} **EPA MRID Number {.....}**

Data Requirement: PMRA DATA CODE: 9.2.4.3 (bee hive)
EPA DP Barcode:
OECD Data Point:
EPA Guideline: 850.3040

Test material:

Common name: Prosper FL (9.49% clothianidin)

Poncho 600 FS (48.0% clothianidin)

Chemical name: (E)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine
IUPAC:
CAS name:
CAS No.:
Synonyms:

Primary Reviewer: Derek François (PMRA) Date:

Secondary Reviewer: Wayne Hou (PMRA) Date:

Company Code: [For PMRA]
Active Code: [For PMRA]
Use Site Category: [For PMRA]
EPA PC Code:

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CITATION: Cutler, C. 2006. An Investigation of the Potential Long-Term Impact of Clothianidin Seed Treated Canola on Honey Bees, *Apis mellifera* L. Department of Environmental Biology University of Guelph (Guelph, Ontario, N1G 2W1); Bayer CropScience; August 1, 2006.

EXECUTIVE SUMMARY:

An investigation on the impacts of clothianidin-treated canola seed on honey bees, *Apis mellifera*, was conducted by allowing bee colonies to forage in canola fields during bloom. Canola seed was treated with clothianidin at a rate of 400 g a.i./100 kg seed, the highest commercial rate for use in Canada. Test sites consisted of a 1-ha field planted with clothianidin-treated seed, and a 1-ha field planted with control canola seed, each separated by at least 250 m. Four honey bee colonies were placed in the middle of each field (n = 32) during a 3-week bloom period, and thereafter moved to a fall apiary. Throughout the study colonies were assessed for bee mortality, worker longevity, and brood development. Samples of honey, beeswax, and worker gathered pollen and nectar were regularly collected and analyzed for clothianidin residues. Colony weight gain while in canola fields and honey yield per colony was also determined.

There were more emergent canola seedlings at 13-14 and 20-21 days post-planting in clothianidin-treated fields than in control fields, however, there was no significant different in later plant development and thus, did not affect the bloom period. The authors reported that bees were actively foraging on canola fields with very little alternative foraging on other crops. There was no significant difference in the weight gain of colonies in clothianidin-treated and control fields. Similarly, there was no significant difference in honey yield from colonies in clothianidin-treated and control fields.

There was a significant difference in worker bee mortality resulting from clothianidin seed treatment at days 77-79 ($P = 0.02$), 92 ($P < 0.001$), 120 ($P = 0.008$) and 127 ($P < 0.001$). All remaining observation dates showed no significant differences in mortality in the clothianidin seed treatment compared to the control seed treatment. In addition, there were no significant differences in the mortality of drones resulting from clothianidin seed treatment compared to the control seed treatment. For many observation dates, mortality in worker and drone bees was higher in the control seed treatment than in the clothianidin seed treatment. Mortality in worker bees was obviously higher in clothianidin-treated colonies at days 13-64 with the DBT. The reported statistical analyses, however, did not reflect this difference and may have been

attributed to the high variability in the number of dead worker bees. Overall, there is no clear indication that clothianidin seed treatment results in the mortality of worker and drone bees.

At no observation time did the amount of sealed brood in colonies from clothianidin-treated fields differ significantly from that found in colonies from control fields. Also, at all observation periods, there was no significant difference in the number of tagged workers found in colonies from clothianidin-treated and control fields.

Results suggest that overall, the effect of clothianidin-treated canola on adult mortality in worker and drone bees is inconclusive as in many cases, mortality in the control bee colonies were relatively high, often greater than that of the clothianidin-treated colonies and given the high variability in the number of dead bees. As a result, there were no significant differences in the weight gain of colonies, honey yield and sealed brood between control and clothianidin-treated colonies.

The study demonstrated, however, that clothianidin residues were detected in pollen, honey and nectar sampled from bee colonies that were exposed weeks before to canola fields grown to clothianidin-treated seed and indicates that clothianidin is translocated to pollen and nectar in canola which then becomes available to foraging bees.

In conclusion, as the control hives were contaminated with clothianidin, the reported results are not valid for the assessment of bee mortality and health of the hive. The contaminated controls may explain why at no observation time did the amount of sealed brood in colonies from clothianidin-treated fields differ significantly from that found in colonies from control fields, or that there was no significant difference in the number of tagged workers found in colonies from clothianidin-treated and control fields. In addition, the study was conducted over a limited time period (3-month) which does not address the overwintering period and subsequent effects on bee colonies in the following year.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED:

COMPLIANCE: The report states that the study was designed and conducted in compliance with good laboratory practices (GLP) as defined in the OECD Principles of GLP (as revised in 1997) and EPA FIFRA GLP Standards (EPA 40 CFR 160).

The report also states that the report is an accurate representation of the raw data as determined by the Quality Assurance inspection which was based on adherence with the study protocol and laboratory standard operating procedures. GLP compliance Quality Assurance and Data Confidentiality were signed.

A. MATERIALS:

1. Test Materials:

Prosper FL

- clothianidin = (E)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine: 9.49%
- thiram = tetramethylthiuram disulfide: 9.49%
- carbathiin = 5,6-dihydro-2-methyl-N-phenyl-1,4-oxathiin-3-carboxamide: 4.43%
- metalaxyl = N-(2,6-dimethylphenyl)-N-(methoxyacetyl)alanine methyl ester: 0.316%

Other Ingredients (%): 76.274%

Poncho 600 FS

- clothianidin = (E)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine: 48.0%

Other Ingredients (%): 52.0%

Description: Flowable Suspension

Lot No./Batch No. : The lot of Prosper FL used (Batch No. 312065M) was from Bayer CropScience, KansasCity, MO. The lot of Poncho 600 FS used (Batch No. 407483M) was from Gustafson, McKinney, Texas

Table 1. Physicochemical properties of clothianidin.

Parameter	Values	Comments
Water solubility at 20EC	327 mg/L	Very soluble in water
Vapour pressure at 20°C	3.8×10^{-11} Pa	Non-volatile
Henry's Law Constant	$1/H = 2.5 \times 10^{13}$	Non-volatile from water and moist soil surfaces
UV absorption	Maximum of 265.5 nm in acidic and neutral solution, maximum of 246 nm in basic solution	Minimal phototransformation is expected in the natural environment.
pKa	11.09	Under acidic and neutral conditions, clothianidin will be in the undissociated form.
Log Kow	0.7	Low potential for bioaccumulation

2. Test organism:

Species: Honey bee, *Apis mellifera*

Age at test initiation: Brood in all stages of development; queens in all colonies were of the same lineage and approximately the same age

B. STUDY DESIGN:

1. Seed Treatment

For the clothianidin treatment, canola seed (*Brassica napus* var. Hyola 420) was treated with a mixture of 1710 g of Prosper FL and 523 g Poncho 600 FS. The mixture was sufficient to treat 100 kg of canola seed resulting in a clothianidin concentration of 400 g a.i./100 kg seed. For the control treatment, canola seed was treated with a mixture of "blank" (without clothianidin) Prosper FL formulation (which delivered the same fungicide treatment) and a "blank" (without clothianidin) Poncho 600 FS formulation. The Gustafson CBT-50 seed treater was used to treat the seed. Table 2 lists the test substances used in the treatment of canola seed with its sources and characteristics. Canola seed was obtained from Interstate Payco Seed Company (West Fargo, North Dakota). Seed treatment was conducted at Gustafson Seed Technology Center (McKinney, Texas) and shipped to the Principal Field Investigator at the University of Guelph (Guelph, Ontario). Samples of control seed and clothianidin-treated seed were sent to ALS Environmental – Edmonton (formally Enviro-Test Laboratories) for verification of clothianidin content.

Table 2. Seed Treatment: Test Substance Characteristics and Sources.

Characteristic/source	Formulation			
	Poncho 600 FS	**Prosper FL**	**Blank Poncho 600 FS**	**Blank Prosper FL**
CAS Number	210880-92-5	210880-92-5 (clothianidin) 137-26-8 (thiram) 5234-68-4 (carboxin) 57837-19-1 (metalaxyl)		137-26-8 (thiram) 5234-68-4 (carboxin) 57837-19-1 (metalaxyl)
Batch / Lot Number	407483M	312065M	TAM113:70-1	TAM113:67-1
Receiving number	N05412	N05415	TAM113:70-1A	TAM113:67-1A TAM113:67-1B
Source	Bayer CropScience	Gustafson	Gustafson	Gustafson
EPA Registration No.	264-789-7501 (clothianidin)	7501-190		
Formulation Type	Flowable suspension	Flowable suspension	Flowable suspension	Flowable suspension
Certified % a.i.	48.96% (clothianidin)	9.64% (clothianidin)		

2. Experimental Design

A total of eight canola fields were established, four on the University of Guelph – Elora Research Station, Elora, Ontario (at 2 sites: E1 and E2) and four on two farms (at 2 sites: W3 and W4), in close proximity to the Elora Research Station. Each site consisted of two 1-ha fields with clothianidin-treated seed planted in one field and control seed planted in the other field. Fields were separated by at least 250 m. Clothianidin-treated and control seed were planted (on May 20-21) at a depth of approximately 4 cm in rows 18 cm apart in [a fine?] firm seedbed at the highest recommended seeding rate of 15-20 seeds/m or 8.0 kg/ha to ensure a high number of plants and, therefore, an abundance of forage for bees. All fields received a single pre-plant herbicide treatment of Treflan EC® (trifluralin) at rate of 960 g a.i./ha and a single fertilizer treatment of 100 kg N/ha as ammonium nitrate.

Seedling emergence rates were determined at 13-14 days and 20-21 days post-planting (on June 3 and June 7-8). On each date, the number of emerged plants per 1 m of row was determined in 10 randomly selected locations in each field. The growth of plants in each 1 m of row (all plants

combined? ***This is unclear – all plants from the 10 locations??)*** was assessed and assigned a rank according to the following: 1 = cotyledon stage; 2 = 2 true leaves; 3 = 3 true leaves; 4 = 4 true leaves; and 5 = 5 true leaves.

Damage to plants from flea beetles (crucifer flea beetle, *Phyllotreta cruciferae* Goeze, and striped flea beetle, *Phyllotreta striolata* (F.)) in each 1 m row was also assessed by assigning a rank according to the following: 0 = no damage; 1 = up to 25% damage; 3 = up to 75% damage; 4 = 100% damage. Plant emergence, development, and flea beetle damage was compared amongst treatments and sites using a general linear model (GLM) statistical platform (SAS Institute 2003). The investigators indicated that, to their knowledge, no other flowering crops (e.g. soybean, corn, alfalfa) or corn grown from seed treated with clothianidin were planted within a 1 km radius of any of the canola test plots and that the availability of this alternative forage to bees was minimal.

After exposure in canola fields, the honey bee colonies were moved approximately 35 km away to a fall apiary (at Cambridge Research Station) for the remainder of the experiment.

3. Colony Establishment

Prior to placement in canola fields, 34 honey bee colonies were held at a spring apiary (near the Townsend House Bee Research Facility, University of Guelph). Thirty-two colonies were used in the trial, while two served as spares. Each colony consisted of a single brood chamber measuring 24 cm deep with 10 frames/super, below a shallow honey super measuring 16.5 cm deep with 9 frames/super. Queen bees in all colonies were of the same lineage and approximately the same age. A queen excluder was placed between the brood chamber and honey super to retain the queen in the brood chamber. Colonies were adjusted to establish similar quantities of food stores (pollen and nectar), brood in all stages of development and number of adult bees. Colonies were assessed for the presence of Varroa mite, tracheal mite, and infectious honey bee diseases (American Foulbrood, European Foulbrood and Chalkbrood) prior to placement in canola fields. Pest and disease status was recorded throughout the study, and where needed, honey supers were added to colonies. All moveable components of each colony (brood chambers, supers, covers, etc.) were labelled to ensure accurate colony-component cross-referencing.

In the middle of each canola field (8 fields in total), a clearing was established by mowing a 10 m x 5 m area to accommodate 4 colonies. Colonies were placed in the clearing of each field when approximately one-quarter to two-thirds of the canola was in bloom (determined by visual

estimation). Colonies were moved at night over a two-day period (e.g. 9:00 pm to 4:00 am). As the bloom period differed slightly in some fields, 8 colonies were moved to 2 fields (W3C and W3T) on June 27/28, while the remaining 24 colonies were moved on June 29/30. Day 0 was designated as June 30, the last day for placement of colonies in canola fields. All colonies were positioned so that colony entrances faced approximately south.

At Days 20 and 21 (July 20 and 21), colonies were removed from canola fields when approximately 20% bloom remained. Colonies were then maintained at the fall apiary until the end of the study on Day 130 (November 7). The colonies from control fields (*n*=16) were separated from those of the clothianidin-treated fields (*n*=16) by at least 30 m within the fall apiary. No other colonies were present at this apiary.

4. Colony Assessment

4.1 Weight Gain

Colonies were weighed on Day 1 and Day 21 using an Easy-Loader Boom scale mounted on the flatbed of a truck. Differences in the weight gain of colonies were compared among treatments (clothianidin-treated and controls) and sites over time using a general linear model (GLM) statistical platform (SAS Institute 2003).

4.2 Honey Yield

Honey yield per colony was determined by weighing empty honey supers before placement on colonies and after removal from colonies using the following equation:

Full Super Weight (removed from colony)	–	**Empty Super Weight** (prior to placement on colony)	=	**Honey Super Yield**

Differences in honey yield (kg/colony) between colonies from untreated and clothianidin-treated fields were analyzed using a general linear model (GLM) statistical platform (SAS Institute 2003).

4.3 Adult Mortality

Bee mortality was assessed using Gary Dead Bee Traps (DBT) or 1 x 2 m white sheet placed on the ground extending out from the hive entrance. The study authors reported that each method has been used effectively in previous studies to assess bee mortality and that, while dead bee losses due to scavengers and wind have been suggested as drawbacks of the entrance sheet method, the placement of colonies in the middle of the canola fields likely reduced exposure to both of these elements.

As only 8 DBT were available, one randomly selected colony at each field was fitted with a DBT, while the entrance sheet method was used for the remaining three (for a total of 8 DBT and 24 sheets). Dead workers and drones were collected from the DBT and white sheets and counted approximately every 7 days from Day 0-130. Collected dead bees were disposed of after counting and sorting. Worker and drone mortality were compared amongst treatments and sites over time using a general linear model (GLM) statistical platform (SAS Institute 2003). Dead bee recovery with DBT and white sheets was also compared statistically.

4.4 Brood Assessment

Assessments of broods were performed on Days -2, 1, 2, 14, 15, 33 and 34 and subsequently approximately every 14 days up to Day 98. The study authors reported that brood assessments were very stressful on the bees as colonies were open for approximately 60 minutes under very hot, no-shade conditions. Coupled with the additional stress on bees of moving the colonies to the fall apiary (a task that took several hours), brood assessments were not conducted on the day of colony removal from canola fields. Alternatively, colonies were allowed to acclimatize to their new surroundings for a week after the move before continuing with the brood assessments.

For each colony, the presence/absence of eggs and unsealed larvae were reported. The area of sealed brood was determined for all colonies. The researchers reported that during the Day 1 brood assessment, they determined that it would not be possible to assess eggs, unsealed larvae, and sealed brood for all 32 colonies. The researchers, therefore, opted to determine the amount of sealed brood only, which they indicate would reflect development of egg and larval stages. The area of sealed brood was estimated using an empty “template” brood frame that was divided into six quadrants. The template was laid over each frame of brood and the percent brood in each quadrant was estimated. This was done on both sides of each frame, for all 10 frames of each colony. Percent estimates were thereafter totalled and converted to cm^2 allowing a determination of the total area of sealed brood per colony. To ensure consistency in estimates, brood

assessments were conducted by the same two individuals throughout the study. Brood area per colony was compared amongst treatments and sites over time using a general linear model (GLM) statistical platform (SAS Institute 2003).

4.5 Worker Longevity

On Day 3, frames of sealed, ready-to-emerge workers were removed from the spare bee colonies located at the spring apiary and placed in an incubator (32 ± 3°C and 60% ± 5% RH) overnight on-site. The following morning, newly emerged workers were marked with OpalithP®colored/numbered thoracic tags (Graze, Bienenzuchtgeräte) and held in small cages with a water source until introduction into one of the field colonies. On Day 4, 50 young, marked worker bees (approximately 24-h old) were introduced to each of the 32 field colonies. In most cases, tag colors were different for each colony at each field, however, in some cases, this was not possible as there were a limited number of colours and associated numbers.

Day 5 assessments determined that tagged worker introductions in some colonies (6 total, 3 control colonies and 3 treatment colonies) were unsuccessful. Therefore, tagged workers – consisting of tags with colours and/or numbers different from those of the initial introduction – were reintroduced to these colonies on Day 8. A second set of tagged workers (colour/number schemes different from first introduction) was added to all colonies approximately halfway through the experiment on Day 70. At that time, 25 colonies had no tagged workers left, 4 colonies had 1 tagged worker, one colony had 5 tagged workers, and a single colony still had 12 tagged workers. Following each reintroduction of tagged workers, those from subsequent introductions were disregarded during data collection. Tagged worker bees were counted on Day 5 and 9 (post-introduction assessments), Day 14 and 15, and thereafter on approximately 14-day intervals up to Day 98. To maximize efficiency and minimize stress on bees, tagged worker assessments were conducted during brood assessments.

Tagged worker longevity, based on the number of tagged workers recorded each collection day, was compared amongst treatments and sites using a general linear model (GLM) statistical platform (SAS Institute 2003).

4.6 Queen Assessments

If located during the brood assessments, queens were visually inspected to ensure normal physical health (e.g. presence of all wings and legs) and behaviour (e.g. laying eggs, surrounded

by retinue of workers). When queens were not located, the presence of eggs confirmed the presence of a laying queen in the colony within the last 3 days.

Inspections of the queen supercedure cells (elongate cells, in the middle or near the top of one or more brood frames, in which a new queen is reared) were conducted in conjunction with the brood assessments. In most cases these cells were opened to verify the presence of a larva, and then destroyed. If the queen was absent in a colony, however, in some cases supercedure cells were left to allow a new queen to be reared by the colony. Swarm cells (queen cells usually found on the bottom of the combs before swarming) when found were also destroyed to prevent swarming.

In some colonies, queens were lost part way through the experiment; e.g. they were accidentally killed during data collection, moving colonies, or rejected by the colony over time. In such cases, marked queens from the same/similar lineage were collected from spare colonies at the spring apiary and introduced to the experimental colonies. In other cases, a new queen was allowed to emerge from a supercedure cell to replace the old queen.

4.7 Residue Analysis

Whenever possible, a total of approximately 5 g of nectar was collected from colonies at each field (pooled samples). Nectar samples were extracted from cells using a disposable syringe, or removed by gently shaking a brood frame over a sheet of waxed paper. Samples were stored in labelled, brown jars, placed in a cooler, and returned to the University of Guelph within approximately 10 hours of collection from colonies. Samples were held in a freezer at –20°C until shipment to ALS Environmental – Edmonton (formerly Enviro-Test Laboratories) for residue analysis. Nectar was collected from colonies on Day -3/-1, Day 7, Day 14/15, Day 42, and thereafter on approximately 21-day intervals up to Day 83.

A total of approximately 5 g of honey from capped cells was collected from colonies at each field (pooled samples) using a small disposable spatula. Honey samples were stored in labelled, brown glass jars, placed in a cooler and returned to the University of Guelph within approximately 10 hours of collection from colonies. Samples were held in a freezer at –20°C until shipment to ALS Environmental – Edmonton for residue analysis. Honey samples were collected on Day -3/-1, Day 7, Day 13, Day 40, and thereafter on approximately 21-day intervals up to Day 102.

Pollen was collected from each colony over a 24-hour period using an OAC pollen trap. A total of approximately 10 g pollen was collected at each field (pooled sample), stored in labelled brown glass jars, placed in a cooler, and returned to the University of Guelph within approximately 10 hours of collection from colonies. Approximately 5 g of pollen was analyzed under a light microscope, which confirmed that bees foraged on canola, while the remainder of sample was held in a freezer at –20°C until shipment to ALSEnvironmental – Edmonton for residue analysis. Pollen was collected on Day -3/-1, Day 7, Day 14/15, Day 42, and thereafter on approximately 21 day intervals up to Day 106.

A total of approximately 3 cm^2 of brood and food-free beeswax was collected from colonies at each field (pooled samples). Samples were sealed in Ziploc® freezer bags, placed in a cooler, and returned to the University of Guelph within approximately 10 hours of collection. Samples were held in a freezer at –20°C until shipment to ALS Environmental – Edmonton for residue analysis. Beeswax was collected on Day -3/-1, Day 7, Day 13, Day 40, and thereafter on approximately 21-day intervals.

All samples were packed in dry ice and sent to ALS Environmental – Edmonton. The content of clothianidin in seed coating was determined using an ALS internal method, 185.01, for formulation on treated seeds. Residue analysis for pollen, honey and nectar was done using the procedures provided by Bayer CropScience: "Residue Analytical Method 00554 for the Determination of Residues of TI 435 in Nectar, Honey, Rape Flower, Rape Pollen and Bee Samples by HPLC with Electrospray MS/MS-detection". Method modification No. MS225.0, “Analysis of Clothianidin (TI 435) in Nectar and Honey by LC/MS/MS, Method”, was employed. For wax, a short summary of methods employed for imidacloprid extraction from beeswax was supplied by Ralf Schoning of Bayer CropScience, Monheim, Germany, and served as the basis for clothianidin extraction from beeswax samples. Sample analyses were completed on May 15, 2006.

4.8 Study Deviations

Reported study deviations are listed in Appendix I. The author reports that these deviations did not have any impact on the study.

5.0 Results

5.1 Seed treatment

Canola seed was successfully treated to within the prescribed rate of 417 g clothianidin/100 kg seed. Clothianidin was not detected in the control seed.

5.2 Canola Emergence

In general, there were significantly more emerged plants per meter in clothianidin seed treated fields than in untreated fields on both sampling days, June 3 (F = 6.07, df = 7, P < 0.0001) and June 8 (F = 4.92, df = 7, P <0.0001); i.e. at 13-14 days and 20-21 days post-planting, respectively. Although there were generally more emerged canola plants/meter in clothianidin-treated fields than in control fields, there was no significant difference in the development of emerged plants. The growth stage of emerged plants were not significantly different with treatment (June 3: χ^2 =1.37, df = 1, P = 0.24; June 8: χ^2 = 0.01, df = 1, P = 0.90) or site (June 3: χ^2 = 1.75, df = 3, P = 0.63; June 8: χ^2 =6.70, df = 3, P = 0.082), and there was no significant difference in the interaction of effects from treatment and site (June 3: χ^2 =1.75, df = 3, P = 0.63; June 8: χ^2 =2.39, df = 3, P = 0.50).

Flea beetle damage was significantly greater in control fields on both June 3 and June 8. Although flea beetle damage did not vary with site on June 3, a difference between sites was found on June 8.

5.3 Weight Gain

There was no significant difference in weight gain of colonies from control and clothianidin-treated fields (F = 0.14, df = 1, P = 0.70). In both treatments (control and clothianidin-treated), colony weights increased approximately 23-24 kg during the three week period. Differences in colony weight gain were not significant amongst sites (F = 2.81, df = 3, P = 0.061), and there was no significant treatment-site interaction (F = 1.02, df = 3, P = 0.40)

5.4 Honey Yield

There was no significant difference in honey yield from colonies from control and clothianidin-treated fields (F = 0.02, df = 1, P = 0.89). A mean of 45.3 kg and 44.7 kg of honey was harvested from colonies in clothianidin-treated and control fields, respectively, over the 130 days. Differences in honey yield were not significant amongst sites (F = 0.45, df = 3, P =

0.72), and there was no significant treatment-site interaction for honey yield (F = 1.22, df = 3, P = 0.33).

5.5 Adult Mortality

The author reported that DBTs (Dead Bee Traps) were occasionally unreliable as on various collection dates, traps were left partially open, came loose from the colony or became partially filled with water after a heavy rainfall. Data from the DBT that were partially filled with water due to rain were, therefore, excluded from the data analysis. In analyzing of number of dead workers and drones, the DBT and paper sheet data were analyzed separately.

The authors reported that, regardless of the dead bee collection method that was used, there were no significant differences in dead worker bees due to clothianidin treatment of canola seed (DBT: F = 0.31, df = 1, P = 0.58; Sheet: F = 0.74, df = 1, P = 0.39) or the day-treatment interaction (DBT: F = 1.07, df = 13, P = 0.40; Sheet: F = 1.14, df = 16, P = 0.32). Similarly, the authors indicated that there was no significant difference in the number of dead drones due to treatment (DBT: F = 1.69, df = 1, P = 0.20; Sheet: F = 0.97, df = 1, P = 0.32) or the day-treatment interaction (DBT: F = 0.95, df = 13, P = 0.51; Sheet: F = 0.31, df = 16, P = 0.99). (*Note: Do not know what these reported values refer to as statistical analyses were performed for each sampling date)*

There were clothianidin seed treatment effects on worker bees at day 77-79 as mortality was significantly different (P = 0.02) from the control seed treatment. Similarly, there was a significant difference in worker bee mortality resulting from clothianidin seed treatment at day 92 (P < 0.001), day 120 (P = 0.008) and day 127 (P < 0.001). All remaining observation dates showed no significant differences in mortality in the clothianidin seed treatment compared to the control seed treatment. In addition, there were no significant differences in the mortality of drones resulting from clothianidin seed treatment compared to the control seed treatment.
For many observation dates, mortality in worker bees was higher in the control seed treatment than in the clothianidin seed treatment. With the DBT method, mortality in the control exceeded that of the the clothianidin treatment at days 71, 77, 89, 92, 112, 120 and 127. With the entrance sheet method, mortality in the control exceeded that of the clothianidin treatment at days 28, 35, 42, 49, 56, 64, 71, 92, 99 and 120. Similarly, mortality in drones was higher in the control treatment at days 28, 49, 56, 64, 71, 77, 89, 92, 106 and 112 using the DBT method and higher at days 7, 13, 18, 28, 42, 49, 56, 64, 71, 89, 92 and 106 using the entrance sheet method. Mortality in worker bees was obviously higher in clothianidin-treated colonies at days 13-64 with the DBT. The reported statistical analyses, however, did not reflect this difference and may have

been attributed to the high variability in the number of dead worker bees. Overall, there is no clear indication that clothianidin seed treatment results in the mortality of worker and drone bees.

5.6 Brood Assessment

At no observation time did the amount of sealed brood in colonies from clothianidin-treated fields differ significantly from that found in colonies from control fields. The observation times were days 1, 14, 33/34, 48/49, 63/64, 76/84 and 97/98.

5.7 Worker Longevity

The number of tagged workers decreased over time in colonies from both clothianidin treated and control canola fields ($F = 307.28$, $df = 5$, $P < 0.0001$). There was no significant effect of site ($F = 0.95$, $df = 3$, $P = 0.42$) or treatment ($F = 0.42$, $df = 1$, $P = 0.51$) on longevity of tagged workers. There also was no significant effects attributed to the interaction of date*site ($F = 0.30$, $df = 15$, $P = 0.99$), date*treatment ($F = 0.47$, $df = 5$, $P = 0.80$), or site*treatment ($F = 1.38$, $df = 3$, $P = 0.25$). At all observation periods, there was no significant difference in the number of tagged workers found in colonies from clothianidin-treated and control fields.

5.8 Other Assessments

It was reported that worker bees were actively foraging on canola in all fields and that very little alternate foraging occurred during the trial.

It was reported that, three colonies were classified as “dead” part way through the study. These colonies were artificially or naturally re-queened during the experiment but failed to successfully establish a queen. Although data from these colonies may have inadvertently been collected (i.e. its status as being “dead” was subsequently realized), these data were omitted from some statistical analyses, e.g. sealed brood analyses near the end of the experiment. The authors concluded that, given adequate data were collected from these colonies through much of the experiment, and that there were a large number of replicates in total, the loss of these colonies had no impact on the study overall.

5.9 Residue Analysis

Clothianidin was detected in clothianidin-treated seed at the prescribed level at an average of 417 g a.i./100 kg seed. Recovery of clothianidin residues from spiked samples was within acceptable limits. In-phase recovery from honey, nectar, pollen, and beeswax was 93%, 89%, 87%, and 104%, respectively.

Clothianidin was detected at concentrations of 0.501-0.928 ng/g in honey collected from colonies in clothianidin-treated canola fields and < LOQ (< 0.5 ng/g) in honey collected from colonies in the control canola fields. Clothianidin was detected at concentrations of 0.521-2.24 ng/g in nectar collected from colonies in clothianidin-treated canola fields and in nectar collected from colonies in the control canola fields at concentrations of 0.535-0.969 ng/g. In pollen, clothianidin concentrations were 0.698-2.59 ng/g obtained from colonies in clothianidin-treated canola fields and < LOQ (< 0.5 ng/g) from colonies in the control canola fields. Clothianidin concentrations in beeswax were < LOQ (< 0.5 ng/g) collected from colonies in clothianidin-treated and control canola fields.

5.10 Conclusions

Results suggest that overall, the effect of clothianidin-treated canola on adult mortality in worker and drone bees is inconclusive as in many cases, mortality in the control bee colonies were relatively high, often greater than that of the clothianidin-treated colonies and given the high variability in the number of dead bees. As a result, there were no significant differences in the weight gain of colonies, honey yield and sealed brood between control and clothianidin-treated colonies.

The study demonstrated, however, that clothianidin residues were detected in pollen, honey and nectar sampled from bee colonies that were exposed weeks before to canola fields grown to clothianidin-treated seed and indicates that clothianidin is translocated to pollen and nectar in canola which then becomes available to foraging bees.

In conclusion, as the control hives were contaminated with clothianidin, the reported results are not valid for the assessment of bee mortality and health of the hive. The contaminated controls may explain why at no observation time did the amount of sealed brood in colonies from clothianidin-treated fields differ significantly from that found in colonies from control fields, or that there was no significant difference in the number of tagged workers found in colonies from clothianidin-treated and control fields. In addition, the study was conducted over a limited time

period (3-month) which does not address the overwintering period and subsequent effects on bee colonies in the following year.

APPENDIX I. STUDY DEVIATIONS

The following SOP deviations made during this study were reported.

Seed Treatment Phase

1. SOP Deviation: Gustafson SOP 4.5 states that two certified weights must be used to check the calibration of the balance before use in a GLP study. These weights must be bracketed by the amount to be weighed. The maximum amount weighed for this phase study was 13.105 kg. The certified weights could not bracket this much weight. The calibration check was conducted with 1 kg (observed = 1.000 kg) and 4 kg (observed = 4.000 kg) standard weights.

2. SOP Deviation: Gustafson SOP 7.2 requires the actual weight of each seed bag be recorded in Section 10 of the SOP in the Seed Treatment Laboratory Notebook. The weights were not recorded as the seed was weighed for the trial.

Field Study Phase

Sixteen Study Plan/SOP deviations were noted during the field study phase of this study. No deviation had any impact on the study.

1. Canola emergence assessment revealed missed plantings in the middle of the control field at site E1. This section of the field was replanted on June 6.

2. The Study Plan states that the first beeswax collection was to be done on DAY -1. In anticipation of time constraints, this was done on DAY -3.

3. The Study Plan states that colonies were to be moved into the canola fields at ca. 20% bloom. This period was missed in fields for site W3, where colonies were placed in fields at ca. 67% bloom.

4. The Study Plan states that colonies were to be moved into the canola fields at ca. 20% bloom. This period was missed in fields for sites E1, E2, and W3. Colonies were placed in these fields at ca. two-thirds bloom.

5. The Study Plan requires nectar to be collected from colonies on DAY -1. This was not possible due to a lack of nectar in the colonies. Nectar was collected from some colonies on DAY -3.

6. The Study Plan indicates that the tag colour for marked workers in the longevity study is to be different for each colony per field. However, there were duplicate colours in colonies in clothianidin-treated fields at site E1, W3, and W4.

7. Introductions of tagged workers at some colonies were unsuccessful on DAY 4. Tagged workers, with different colour and number schemes, were therefore reintroduced to colonies W3Cb, W3Cc, E1Tc, W4Cb, E1Tb, and W3Td on DAY 8.

8. The Study Plan indicates that colonies are to be moved into the canola fields on DAY 0. However, due to uneven initiation of bloom in fields, all colonies were not moved into fields on the same day; 8 colonies were moved into fields at site W3 on June 27/28 (from ca. 10:00 pm on June 27 to 1:00 am on June 28), while 24 colonies were moved into fields at sites E1, E2 and W4 on June 29/30 (from ca. 10:00 pm on June 29 to 3:00 am on June 30). June 30, the date colony moving was complete, was identified as Day 0. Therefore, 8 colonies were moved on DAY -2 instead of DAY 0.

9. SOP CSD-036 states that during collection of nectar, honey, pollen and beeswax samples, new collection vessels (jars or plastic bags) and sampling tools (syringes or spatulas) will be used for each colony. However, since samples were pooled amongst the 4 colonies in each field, the same syringe/spatula and sample jar/plastic bag was used for each sample in a given field (e.g. one spatula for honey collection per all 4 colonies per field).

10. The Study Plan states that honey and beeswax sample collections, and dead bee assessments are to be conducted on DAY 14. Due to time constraints, these data were acquired on DAY 13.

11. The Study Plan states that brood assessment, and nectar and pollen sample collections are to be conducted on DAY 14. Due to time constraints, these data were acquired on DAY 14 for colonies at sites E1 and E2, and on DAY 15 for colonies at sites W3 and W4.

12. The Study Plan states that nectar is to be collected on DAY 7. Nectar was not

collected from all colonies on DAY 7 since it was not present in all. Nectar was collected from at least one colony per field. A Protocol Amendment (No. 2) was prepared to reflect the inability to collect nectar from all colonies on all sampling days.

13. The Study Plan states that pollen, nectar, honey and beeswax samples are to be placed in a -20°C freezer within 4 h of collection. Given the amount of time required to collect all samples, placement of samples in the -20°C freezer within 4 h was not possible. Since this was the case on all collections, a Protocol Amendment (No. 1) stating that samples were to be returned to the -20°C freezer within 10 h of collection.

14. Sections 4.0.1 of the Study Plan states that "Marked worker bees will be counted on Day 14, Day 24, the day of colony transfer from canola fields to the fall apiary, and subsequently on ca. 10 day intervals until no marked bees are found, or until Day 130, whichever comes first." The following deviations occurred:

a. Tagged worker assessments were conducted during brood assessments on approximately 14 day intervals instead of 10 day intervals;

b. Tagged workers were not assessed the day of colony transfer from the canola fields; and

c. Although some colonies still had tagged workers present, a second tagged worker introduction was done in all colonies DAY 70.

To maximize efficiency and minimize stress on bees, tagged worker assessments were conducted during brood assessments on ca. 14 day intervals. As with the brood assessment, we decided not to assess tag workers on the day of colony transfer from the canola fields in order to minimize stress to the bees.

Up to DAY 63, 6 colonies still had at least 1 tagged worker found from the original introduction on DAY 4. However, since the vast majority of colonies (26 of 32) had no tagged workers at this time – ca. the half-way point of the experiment – we felt that a second introduction was justified on DAY 70.

15. Sections 5.1 of the Study Plan states that "The nectar sample will be removed directly from marked sections of comb using a syringe." Most often, nectar was

collected using a shake method (SOP CSD-036), whereby a brood chamber frame containing nectar was shaken over a piece of waxed paper. The nectar was then poured into a sample jar. The syringe method proved to be very timing consuming and less efficient than the described frame shaking method.

16. Sections 5.0 of the Study Plan states for nectar, honey, pollen, and beeswax collection, that samples "… will be collected from the 4 colonies at each field (pooled samples)." However, collection of samples from all 4 colonies at a field was not always possible and did not always occur.

17. Sections 3.9.4, 4.0.1, and 5.0 of the Study Plan state that data collections for brood assessments, worker longevity, and residue analysis, respectively, will be conducted up to the end of the experiment (DAY 130). However brood assessments and worker longevity counts were halted on DAY 98. Nectar collection was halted after DAY 85. Honey, pollen and beeswax collection were halted after DAY 102. Data collections were halted as colonies were prepared for overwintering on DAY 104.

18. Brood and tagged worker (longevity) assessments were to be done on all colonies on approximately 14 day intervals (see Amendment No. 4 and Deviation No. 14). However, due to a lack of personnel, only half of the number of colonies could each be assessed during the weeks of DAY 77 and DAY 88.